Supporting Information

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SI Materials and Methods

Antibodies. Antibodies against Hap1 and Ahi1 were generated in our previous studies (1). Other antibodies used in the study were obtained from commercial sources as follows: mouse anti-β-tubulin and anti-biotin (Sigma-Aldrich); mouse anti-TrkB and β-catenin (BD Biosciences); rabbit anti-p-TrkB (Epitomics), rabbit anti-Akt, rabbit anti-p-Akt, mouse anti-p-Erk, and rabbit mAb TrkB (80G2) (Cell Signaling Technology), rabbit anti-Erk2 (Santa Cruz Biotechnology), mouse anti-Hrs (Alexis), anti-Lamp1 (Developmental Studies Hybridoma Bank).

Western Blotting Analysis. The brain tissues were quickly collected and homogenized. After protein extraction and determination of concentration, proteins (10–50 μg) were electrophoresed and electrotransferred to nitrocellulose membrane. Blots were incubated with primary antibodies overnight at 4 °C. After washing with 1× PBS and blocking with 5% milk in 1× PBS, blots were incubated with HRP-conjugated secondary antibody (Jackson Immunolaboratories) for 1 h, followed by developing with the ECL Plus Western Blotting Detection System (GE Healthcare). Chemiluminescence signals were captured on autoradiographic blue films (Denville Scientific). Films were scanned and densitometric analysis of the bands was performed with AlphaEase Image Analysis Software (Version 3.1.2, Alpha Innotech).

Fluorescence Immunostaining. Fresh brain cryosections (10 μm) were mounted on gelatin-coated slides and fixed in 4% paraformaldehyde in PBS for 10 min at 25 °C. This procedure was followed by the incubation with blocking buffer (10% goat serum, 3% BSA, and 0.3% Triton-X 100 in PBS) for 1 h. The primary antibodies against Ahi1 (rabbit anti-GST-N-terminal mouse Ahi1) and Hap1 (guinea pig anti-Hap1, EM77) were diluted (1:1,000) in blocking buffer and incubated at 4 °C overnight. For secondary antibodies, Donkey anti-rabbit IgG conjugated with Alexa 488 (1:1,000, Molecular Probes) and rhodamine red-X-conjugated AffiniPure donkey anti-guinea pig IgG (1:1,000, Jackson ImmunoResearch Laboratories) were incubated with tissues for 30 min at 4 °C. Sections were counter-stained with Hochester (1:2,000, Molecular Probes), which was applied together with the secondary antibodies. Fluorescent images were acquired on a Zeiss microscope (Axiovert 200 MOT; Carl Zeiss Imaging) equipped with a digital camera (Hamamatsu Orca-100) and OpenLAB software (Improvision Inc). We used 10×, 40×, and 63× objectives for image acquisition.

Immunoprecipitation. Brainstem tissue from the control and Ahi1-deficient mice were cut into 300-μm slices and incubated with artificial cerebrospinal fluid (ACSF) at 37 °C for 1 h for slice recovery as above. The slices were treated with BDNF (100 ng/mL) at 37 °C for 30 min and homogenized in lysis buffer. Homogenates were centrifuged at 160,000 × *g* for 15 min, and the supernatants were used for immunoprecipitation. Samples were adjusted to 500 μl at 1 μg of protein/μL and preabsorbed by protein A agarose beads (100 μL; 1406–5G; Sigma-Aldrich) for 2 h at 4 °C with gentle rocking. The precleared supernatants were incubated with 5 μL of mouse anti-Hrs antibody at 4 °C overnight with gentle rocking. Protein A beads (50 μL) were added and incubated for 2 h at 4 °C. Samples were spun for 5 min at 2,000 × *g* at 4 °C. Beads were further washed with lysis buffer three times, and the bound proteins were eluted with 1× SDS sample buffer. The immunoprecipitated proteins were subjected to Western blot analysis for the detection of TrkB, Hap1, and Hrs.

Sucrose Density Gradient Fractionation. Brains of *nes-Ahi1*$^{+/-}$, *nes-Ahi1*$^{-/-}$ mice, wild-type or Hap1 null mice were homogenized in a buffer (100 mg/mL) containing 20 mM Hepes, 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM Mg Cl2, 1.53 mM KH_2PO_4, 212.7 mM sucrose, 0.5 mM DTT, 100 μg/mL PMSF, and 1× protease inhibitor mixture (Pierce Biotechnology). Postnuclear supernatant was prepared by centrifugation of the homogenized brain lysates at 1300 × *g* for 5 min. A 5–45% (wt/vol) sucrose gradient was formed in a 12-mL tube containing 5% and 45% sucrose solutions with 10 mM Hepes, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 100 μg/mL PMSF, and 1× protease inhibitor mixture. One milliliter of the postnuclear supernatant was loaded and centrifuged at 35,000 rpm in a Beckman SW41 rotor for 120 min at 4 °C. A total of 12 fractions of 1 mL each were collected and subjected to SDS electrophoresis and immunoblotting analysis. The samples were separated on a 4–12% Tris-glysine SDS-polyacrylamide gel (Invitrogen) for Western blot analysis.

Forced Swim Test. The forced swim test is typically used to examine depressive behavior in rodents. Mice were placed individually into a round plastic cylinder (18 cm height, 15 cm diameter) filled with water (25 °C) at a depth of 10 cm. The mice were rated for immobility, and the immobility time, defined as floating or the absence of active behaviors such as swimming or struggling to escape, was measured. Slight movements of the feet and tail necessary to keep the head above water were excluded as mobility. The immobility time was measured for 6 min by an investigator who was kept blind to the genotypes of the mice and drug treatment. No pretest training of mice was performed.

Tail Suspension Test. The tail suspension test is another widely used model for assessing antidepressant compounds in mice; it is considered as efficient as the forced swim test for predicting antidepressant efficacy. Immobility was rated in the tail suspension test according to the previous description. Briefly, mice were suspended by taping the tail (≈1 cm from tip of tail) to a horizontal wood bar at a height of 40 cm from the table surface for 6 min. The trial was performed for a period of 6 min and the immobility time was recorded manually via stopwatch by a trained observer who was blind to the genotypes of the mice examined. Mice were considered immobile when hung passively and motionlessly without escape-oriented behaviors.

Rotarod Test. Movement coordination performance was evaluated using an AccuRotor rotarod apparatus (Accuscan Instruments). Mice were trained on 2 consecutive d for three 5-min trials at 5 rpm. Testing was performed at the third day. During testing, the rotating rod was set to accelerate from 0 to 40 rpm in 5 min. Each mouse performed three trials on testing day with 5-min resting periods between each trial. Latency to fall from the rotating rod was measured and averaged for the three trials.

Locomotor Activity. Locomotor activity was assessed with an automated system (San Diego Instruments) with photobeams that recorded ambulations (consecutive beam breaks). Mice were placed individually in the chambers with free access to food and water in a 12-h light (0700–1900 hours) and 12-h dark (1900–0700 hours) cycle. Mice were allowed to acclimate for 3 h before recording. Activity was recorded once per hour.

Surface Receptor Cross-Linking Assay in Vivo and in Vitro. The surface TrkB receptor level was examined with modification by using the membrane-impermeable cross-linking reagent bis(sulfosuccinimidyl)

substrate (BS^3), which reacts efficiently with primary amino groups (-NH_2) in cell membrane proteins, as described previously (2). For in vivo assays, brain tissue from control (*nes-Ahi1*$^{+/-}$) and Ahi1-deficient (*nes-Ahi1*$^{-/-}$) mice were sliced to a thickness of 300 μm by a vibration microtome. The brainstem was dissected and incubated in ACSF at room temperature for 1 h for slice recovery. The samples were treated with either BDNF (100 ng/mL, ProSpec) in ACSF or in ACSF only for 1 h. The samples were incubated with 2 mM BS^3 (Pierce) with gentle agitation for 1 h at 4 °C. The samples were then quenched with 20 mM glycine for 10 min and washed in cold ACSF. The samples were homogenized and subjected to SDS/PAGE. For in vitro assay, primary brainstem neurons were cultured from control (*nes-Ahi1*$^{+/-}$) and Ahi1-deficient (*nes-Ahi1*$^{-/-}$) embryonic (E17) mice for 8 d. The cultured neurons were then treated with BDNF and BS^3 in the same manner as the in vivo assay.

Internalization of Biotinylated BDNF. Internalization of biotinylated BDNF assay was performed by using a previously described method (1). Cultured primary brainstem cells were infected with either scrambled or Ahi1 shRNA virus (1) at 7 d in vitro (DIV) for 72 h. Internalization of biotinylated BDNF assay was carried out at 10 DIV. BDNF (2 μg) was biotinylated with no-weigh sulfo-NHS-LC-Biotin (0.1 mg, Pierce) for 30 min at room temperature. Unbiotinylated BDNF-biotin was removed by desalt spin columns (Pierce). Biotinylated BDNF was incubated with cultured brainstem cells on ice for 30 min. The brainstem cells were washed twice with culture medium to remove unbound BDNF. Internalization was initiated by incubating the cells with warm media (37 °C) for 30 min. Surface-bound BDNF was quenched by ice-cold PBS containing 100 mM glycine, and the cells were fixed with 4% paraformaldehyde in PBS. The internalized BDNF-NHS-LC-Biotin was visualized by anti-biotin antibody (1:10,000, Sigma) and immunofluorescence staining.

TrkB Receptor Degradation Assay. Sliced brainstem tissues from the control and Ahi1-deficient mice were incubated with ACSF at 37 °C for 1 h for recovery and then were treated with BDNF (100 ng/mL) for different times. After BDNF treatment, the tissues were subjected to lysis buffer and SDS/PAGE. For densitometric analyses, TrkB level was normalized by loading control β-tubulin and expressed as the percentage of TrkB level without BDNF treatment. Each group contained four mice.

1. Sheng G, et al. (2008) Huntingtin-associated protein 1 interacts with Ahi1 to regulate cerebellar and brainstem development in mice. *J Clin Invest* 118:2785–2795.

2. Mao LM, et al. (2009) Stability of surface NMDA receptors controls synaptic and behavioral adaptations to amphetamine. *Nat Neurosci* 12:602–610.

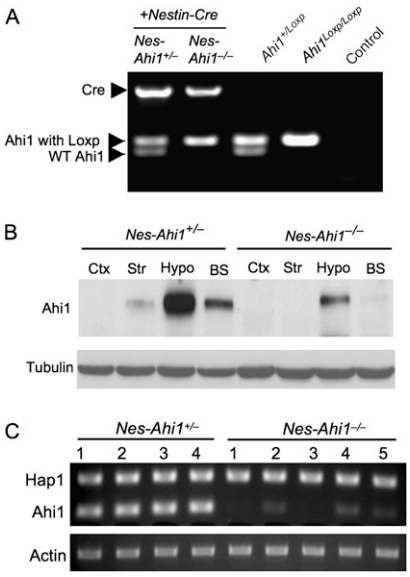


Fig. S1. Generation of conditional *Ahi1* knockout mice. (*A*) DNA samples were extracted from the tails of mice of various genotypes as indicated. The control is PCR without genomic DNA template. PCR was performed with Cre and loxP primers, and PCR products were revealed on 2% agarose gel. (*B*) Western blot analysis of Ahi1 expression in the cortex (Ctx), striatum (Str), hypothalamus (Hypo), and brainstem (BS) of heterozygous (*Nes-Ahi1*$^{+/-}$) and homozygous (*Nes-Ahi1*$^{+/-}$) conditional knockout Ahi1 mice that express Cre under the control of the nestin promoter. (*C*) RT-PCR did not reveal any changes in Hap1 mRNA in Ahi1-deficient (*nes-Ahi1*$^{-/-}$) mice. The numbers (four or five) of mice of each genotype used to isolate the brain cortex mRNAs for RT-PCR are indicated.

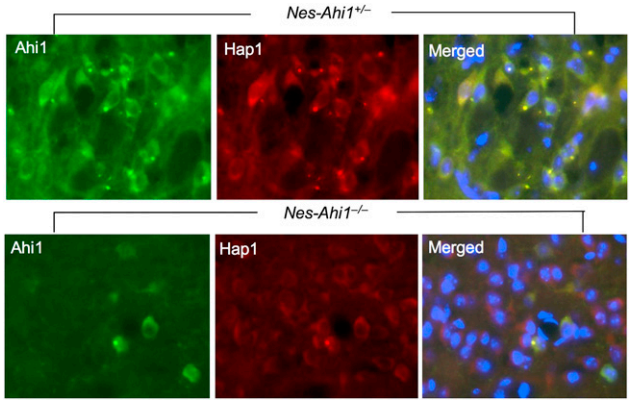


Fig. S2. Ahi1 deficiency diminishes Hap1. Immunocytochemical analysis of the hypothalamic sections from control (*nes-Ahi1*$^{+/-}$) mice and Ahi1-deficient (*nes-Ahi1*$^{-/-}$) mice. Ahi1 (green), Hap1 (red), and nuclei (blue) are seen in the merged images.

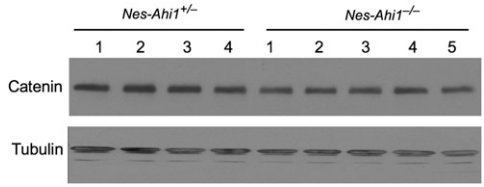


Fig. S3. Western blot analysis of the levels of catenin in Ahi1-deficient (*nes-Ahi1*$^{-/-}$) mice. The controls are heterozygous conditional knockout mice (*nes-Ahi1*$^{+/-}$). The numbers of individual mice for each group are indicated. The same blots were also probed with the antibody to tubulin.

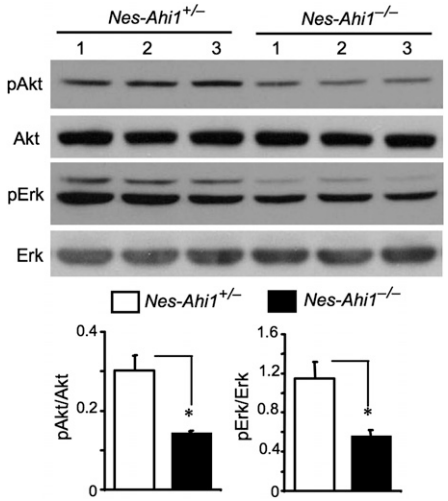


Fig. S4. Ahi1 deficiency reduces TrkB signaling. Western blotting shows the expression levels of Akt, Erk, and their phosphorylated forms (pAkt and pErk) in the hypothalamus of control (*nes-Ahi1*$^{+/-}$) mice and Ahi1-deficient (*nes-Ahi1*$^{-/-}$) mice. Densitometric analysis of the ratios of pAkt to Akt or pErk to Erk showing a decrease of TrkB signaling in Ahi1-deficient (*nes-Ahi1*$^{-/-}$) mice ($n = 3$ each group, $^*P < 0.05$).

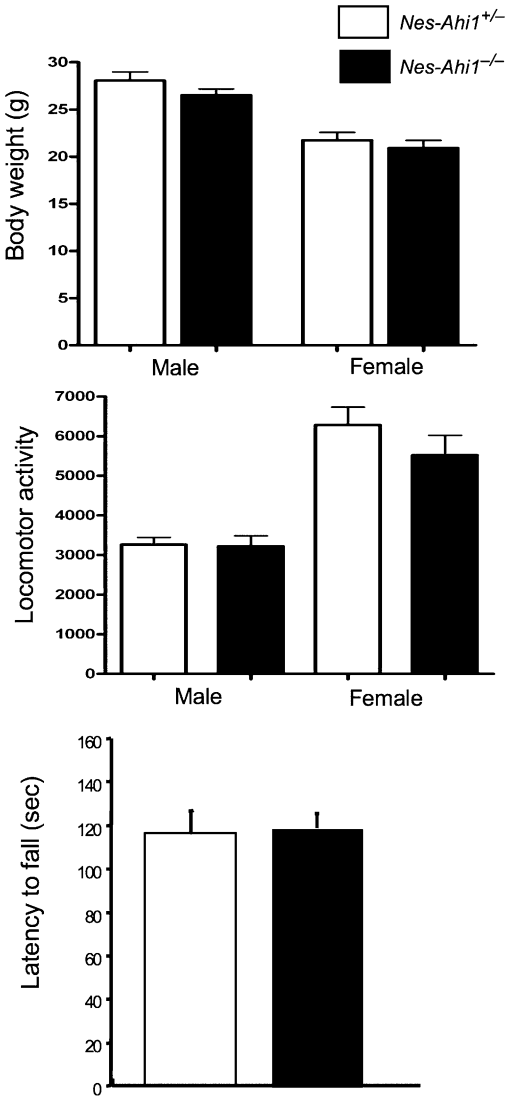


Fig. S5. The body weight and motor function of conditional Ahi1 knockout mice. The body weight, locomotor activity, and rotarod performance of littermate control (*nes-Ahi1*$^{+/-}$) and Ahi1-deficient (*nes-Ahi1*$^{-/-}$) mice. For the rotarod performance test, mice were trained on a rotarod device for 2 consecutive d. Latency to fall from the rod was recorded on the third day as an indication of movement coordination. Mice at the age of 4–6 mo were examined. n = 11–14 for each group.

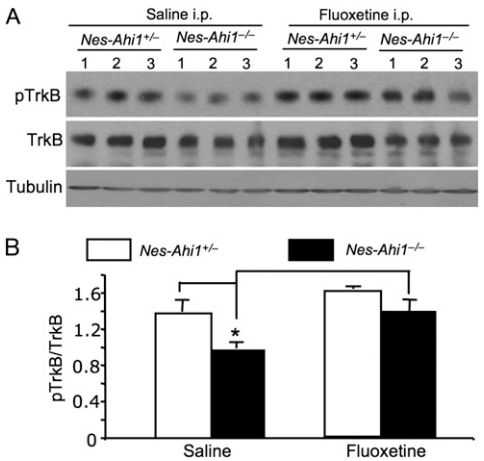


Fig. S6. Overexpression of TrkB in the amygdala of Ahi1 mutant mice. (*A*) Western blot analysis of the brain amygdala tissues of control (*Nes-Ahi1*$^{+/-}$) and Ahi1 knockout (*Nes-Ahi1*$^{-/-}$) mice that had been injected with fluoxetine for 30 min. The same blot was sequentially probed with antibodies to phosphorylated TrkB (pTrkB), total TrkB, and tubulin. (*B*) Densitometric analysis of the ratio of pTrkB to total TrkB. The data were presented as mean + SEM. *$P > 0.05$.

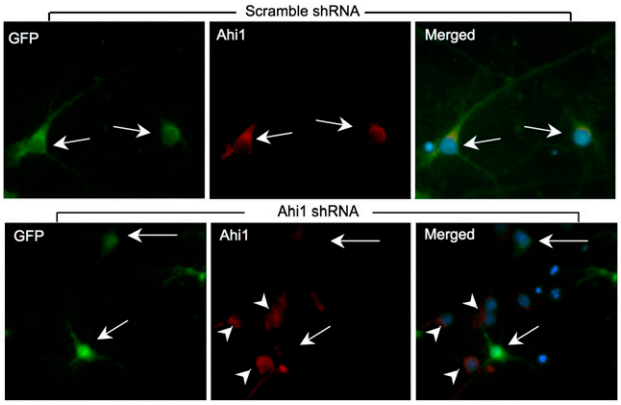
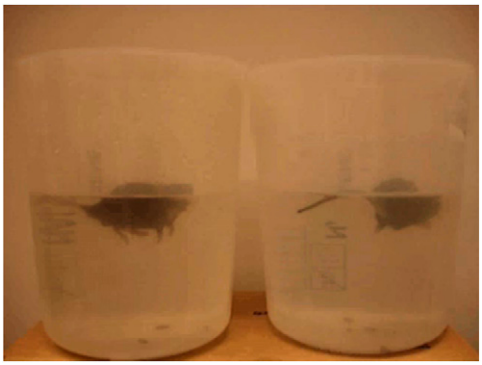


Fig. S7. Immunofluorescent staining of cultured brainstem cells treated with adenoviral Ahi1 shRNA. The viral vector coexpressing adenoviral Ahi1 shRNA and GFP was used to infect mouse brainstem cells at 7 DIV for 72 h. Ahi1 immunostaining (red) shows that control viral vector expressing GFP and scrambled shRNA did not reduce Ahi1 (red) in the infected neurons (arrows in *Upper*) whereas decreased Ahi1 was seen in viral Ahi1 shRNA infected neurons (arrows in *Lower*). Note that uninfected neurons (arrowheads in *Lower*) show positive Ahi1 staining.

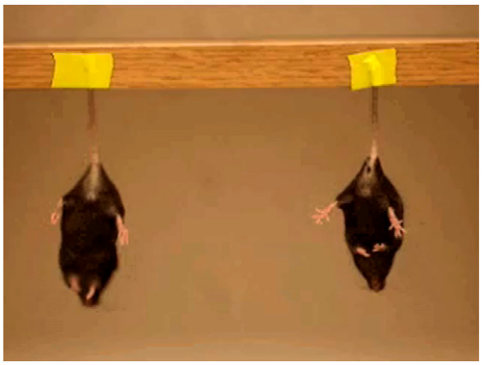


Movie S1. Forced swim test of male control (*Left*, E497, *nes-Ahi1*$^{+/-}$) and Ahi1 mutant (*Right*, E498, *nes-Ahi1*$^{-/-}$) mice. The video was recorded for 54 s.

Movies S1

Movie S2. Forced swim test of male control (*Left*, E511, *nes-Ahi1*$^{+/-}$) and Ahi1 mutant (*Right*, E514, *nes-Ahi1*$^{-/-}$) mice. The video was recorded for 59 s.

Movies S2



Movie S3. Tail suspension test of male control (*Left*, E235, *nes-Ahi1*$^{+/-}$) and Ahi1 mutant (*Right*, E247, *nes-Ahi1*$^{-/-}$) mice. The video was recorded for 50 s.

Movies S3